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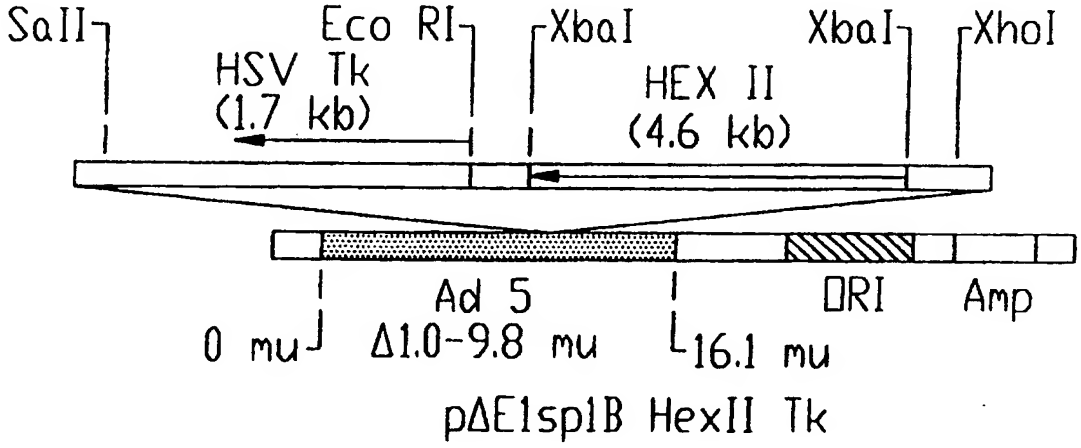
(54) Title: HEX II TUMOR-SPECIFIC PROMOTER AND USES THEREOF IN CANCER THERAPY



(57) Abstract

The present invention relates to a tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II promoter. The present invention also relates to a gene construct, which include Hex II promoter in a vector selected from pCAT basic expression vector pΔE1splB and a shuttle plasmid, and which optionally includes β-gal or HSV Tk.

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HEX II TUMOR-SPECIFIC PROMOTER AND USES THEREOF IN CANCER THERAPY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, such as to drive a suicide gene in cancer therapy.

(b) Description of Prior Art

A successful gene therapy approach is dependent upon two parameters: 1) efficiency of target cells transduction and 2) specificity of gene delivery. Selective targeting is especially critical in the context of cancer therapy for gene directed enzyme prodrug therapy (GDEPT), where a suicide gene expressed in tumor cells encodes an enzyme that converts an otherwise non-toxic prodrug into its active form.

Several methods have been explored to increase the specificity. They can be broadly divided into two categories: directed delivery of the gene of interest or its directed expression. The ideal candidate for transcriptional targeting would be a tumor specific promoter and/or enhancer and its activation will be strong enough to achieve therapeutic levels of the desired transcript. A wide range of promoters have been explored in this context. They were mostly characterized as tissue specific promoters as opposed to tumor selective. Some examples are: surfactant protein SP-A promoter for non small cell lung carcinoma (NSCLC), immunoglobulin enhancer or O enhancer for B-cell lineage cancers, tyrosinase for melanomas, and MUC-1/Df3 for breast cancer. However, these promoters also direct gene expression in the normal tissue of origin of these neoplasms and other critical organs as

well. The erbB2 and a-fetoprotein promoters are activated to a greater extent in certain neoplasms. They have also been used in this strategy and have lead to promising results. Nonetheless, other promoters to further improve and optimize this strategy are needed.

A striking characteristic of rapidly growing tumor cells is their high rate of glucose utilization compared to their normal counterparts. Glucose is mainly channeled through the glycolytic pathway which is not only used for rapid energy production but also for the provision of biosynthetic precursors necessary to sustain a high rate of cellular division. Hexokinase (ATP: D-hexose-6-phosphotransferase) catalyses the first committed step of glycolysis; therefore it was suspected by many to be a potential player in this phenotype. Hexokinases (HK) are comprised of two highly homologous 50kDa halves and are product inhibited by glucose-6-phosphate to varying degrees. They exist in four molecular forms, HK I to HK IV, with distinct electrophoretic and kinetic properties (Wilson, J.E., (1985) *In Regulation of Carbohydrate Metabolism*, Vol I, 45-85, CRC Press, Boca Raton). The profile of these enzymes in tissues at different stages of malignancies shows an increase in HK II in tumor versus normal tissues. In rats, the type I HK is expressed in brain, kidney and heart. The type II HK was found in skeletal muscle and in AH130 hepatoma cells. In normal liver it is type IV HK that is most abundant (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

Comparison of the rat hexokinase II with a hexokinase from rat Novikoff ascites shows there is a single type II isozyme that is found in both normal and tumor tissues (Adams, V., Kempf, W, Hassam, S., and Briner, J. (1995) *Biochem. Mol. Med.* **54**, 53-58). The

inhibition of HK II by glucose-6-phosphate is delayed. Therefore, tumors are able to build up high levels of this product. Its accumulation is a signal for glucose availability for consumption, a stimulus of biosynthetic pathways for growth (Wilson, J.E., (1985) *In Regulation of Carbohydrate Metabolism*, Vol I, 45-85, CRC Press, Boca Raton). The level of HK II was also found to be increased in human HepG2 cells and in renal cell carcinoma (Adams, V., Kempf, W, Hassam, S., and Briner, J. (1995) *Biochem. Mol. Med.* **54**, 53-58). Two factors are involved in this increased activity: the propensity of the tumor enzyme to bind to the outer mitochondrial membrane (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925) and overproduction of the enzyme. The latter is due to both a gene amplification of the tumor type II isozyme and to its transcriptional upregulation (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925). The promoter for the rat tumor type II enzyme has recently been cloned. Regulation of the promoter with known modulators of glucose metabolism was found to be different in hepatoma cells and normal rat hepatocytes (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

It would be highly desirable to be provided with a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells compared to normal cells.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a novel tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer

cells, such as to drive a suicide gene in cancer therapy.

In accordance with the present invention there is provided a tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II reporter gene.

In accordance with the present invention there is also provided a Hex II gene construct, which comprises Hex II promoter in a vector selected from pCAT basic expression vector pΔElsplB and a shuttle plasmid.

In accordance with one embodiment of the present invention the gene construct further comprises β-gal or HSV Tk.

In accordance with another embodiment of the present invention, the preferred gene construct based on pCAT vector is pHexII4557-CAT.

In accordance with another embodiment of the present invention, the preferred gene constructs based on pΔElsplB are pΔElsplBHex-LacZ and pΔElsplBHex-TK.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the Hex II reporter gene construct in pCAT basic expression vector in accordance with the present invention;

Fig. 2 illustrates the Hex II promoter construct including β-galactosidase in the shuttle plasmid pΔ ElsplB in accordance with the present invention;

Fig. 3 illustrates the Hex II promoter construct including HSV Tk in the shuttle plasmid pΔElsplB in accordance with the present invention;

Fig. 4 illustrates a graph of the results of MUC-1 versus HexII promoters activation in normal bronchial and mammary epithelial cells; and

Fig. 5 illustrates a graph of the results of HexII promoter activation in normal bronchial epithelial cells versus non-small cell lung carcinomas.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a new HexII promoter. Its constructs are illustrated in Figs. 1 to 3.

1. Construction of recombinant plasmids

pHexII4557-CAT

(8.9 kb) The HexII, 5.15 kb, promoter in the plasmid pUC18 (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925) was released with an XbaI digest and cloned into the pCAT basic vector (Promega). The size of the promoter was reduced to 4.56 kb with a BamHI digest that released sequences from the non coding region at the 3' end of the clone.

pΔElsplBHex-LacZ

(14.7 kb) the 3.74 kb lacZ gene (HindIII-SalI) from pSV2-β-galactosidase was cloned into the HindIII and SalI polycloning sites of the shuttle vector pΔ ElsplB. This shuttle plasmid contains Adenovirus 5 (Ad5) sequences from map unit 0 to 1, followed by the polycloning site, followed by Ad5 sequences from mu 9.8 to 15.8, and therefore allows recombination to take place with the adenoviral genome. The Hex II promoter 4557 bp was released from the pHexII 4557/CAT with XbaI followed by an EcoRI digest and cloned into the XbaI site of the pΔElsplB. Clone 10 (pΔElsplBHexII) that had the insert in the negative orientation relative to the polycloning site of the pΔElsplB was used for further cloning of the Hex LacZ plasmid. pΔElsplBLacZ was digested with XhoI followed with a partial digest with

EcoRI. pΔElsplBHexII was in turn digested with XhoI and EcoRI, and the purified 4.6 kb fragment was ligated into pΔElsplBLacZ.

pΔElsplBHex-TK

(12.6 kb) The 1.7 kb HSV-TK gene (EcoRI-SalI) from pMClTK was cloned into the corresponding sites of pΔElsplB. Subsequently, the resulting pΔElsplBTK plasmid was cut with EcoRI and XhoI, and the purified 4.6 kb HexII fragment with compatible ends was ligated into it. Plasmid DNA was purified by alkaline lysis followed by cesium chloride density gradient purification.

The use of tissue or tumor selective promoters in targeted gene therapy for cancer depends on strong promoters with specific activity. The Muc-1/Df3 promoter has been used in the context of gene directed enzyme prodrug therapy (GDEPT) (Chen et al (1995) *J. Clin. Invest.* **96**(6), 2775). However we have found that it has limited promoter activity and appears to be expressed in a wide range of normal cells (Fig. 4). An interesting property of cancer cells that could be exploited to target them selectively is their increased rate of glycolysis. Hexokinase type II (Hex II) catalyzes the first committed step of glycolysis and has been linked to this phenotype since it is overexpressed in tumors and is not responsive to the normal physiological inhibitors, e.g. glucagon (Mathupala, S.P., Rempel, A., and Pedersen, P.L. (1995) *J. Biol. Chem.* **270**, 16918-16925).

In accordance with the present invention, the tumor HK II promoter was tested in variety of human tumor cell lines and in normal human cells. We studied the Hex II promoter by transfecting cells with the pHex II4557/CAT (Fig. 1) construct and performing a

chloramphenicol acetyl transferase (CAT) reporter gene assay.

2. Transfection and reporter gene assays

Transient transfections were performed using lipofectamine according to the manufacturer's recommendations (GIBCO-BRL). Cells were plated the day before transfection to give 60% confluency in 6-well plates. The pl583/+33MUC1.CAT or pHex4557.CAT vectors were transfected along with pSV_2lacZ to determine promoter activity. 1 ug of each plasmid were used for each well. All conditions assayed were done in duplicate. The plasmids pRSV.CAT and promoterless pCAT were used as positive and negative controls, respectively. Cells extracts were prepared 48 hours after transfection and β-galactosidase activity was assayed to compensate for variations in transfection efficiency. CAT activity was determined from 75-100 ug of proteins. The reaction was carried out with 0.1 uCi of ^{14}C-labeled chloramphenicol in a 100 ul reaction at 37°C for 4 hrs.

Results

Its activation was very high in tumor as opposed to normal cells. The activation of HeX II in the non-small cell lung carcinomas H661 and H460 was 43% and 64% (respectively) of the activation observed with the Rous Sarcoma virus (RSV) constitutive promoter while it was 3% of RSV in the primary normal human bronchial epithelial cells (NHBEC). Moreover, treatment of the transfectants with glucagon did non inhibit promoter activation in H661 cells. Its activation in the human mammary carcinoma cells MCF-7 was 72% of RSV while it was 23% of RSV in the normal human mammary epithelial cells (NHMEC).

Moreover, the efficacy of this promoter in the context of GDEPT was tested by using the herpes thymidine kinase gene in combination with the prodrug gancyclovir.

The following suicide genes may be used in accordance with the Hex II promoter constructs of the present invention: Cytochrome P-450™ 2B1 with cyclophosphamide, penicillin, amidase and β-lactamase.

3. MTT cell viability assays

Cell survival was determined using a colorimetric assay which measures the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to an insoluble purple formazan precipitate. Cells in the logarithmic phase of growth were resuspended at a concentration of $2x10^5$ cells/ml. 2ml/well were plated in 6-well plates. Plates were incubated for 24 h at 37°C in 5% CO_2. Subsequently, cells were transfected with the pΔElspIB Hex TK plasmid as described above. 6 h after the transfection, cells were treated with the drug gancyclovir at concentrations of 10 or 25 ug/ml. Each condition was done in triplicate. Cells survival was calculated in the treated population as a percentage of controls. Controls are cells transfected with the plasmid alone or treated with the drug alone. MTT assays was performed two days following treatment. The formazan crystals were dissolved in dimethyl sulfide (Fisher) and glycine buffer (0.1 M glycine- 0.1 M NaCl, pH 10.5). The formazan product formed by viable cells was quantitated by measuring the absorbance at a wavelength of 570 nm.

Results

Cell survival in the transfectants exposed to gancyclovir (GCV) at doses of 10 or 25 ug/ml was 50%

less than control cells treated with GCV alone or transfected with the plasmid only. We are presently examining the potential use of Hex II-VTK in recombinant Ad5 in the treatment of tumor bearing animals.

The regulation of this promoter in human tumor cell lines was studied using glucose, insulin, and glucagon. Lack of metabolic repression was confirmed as described by Mathupala, S.P. et al. ((1995) *J. Biol. Chem.* **270**, 16918-16925). In addition, several samples of human tissues were screened with the HK I, HK II, and HK IV cDNAs to evaluate the level of these enzymes in tissues and asses the safety of using this promoter in gene therapy.

We hypothesize that the Hex II promoter, with or without the metabolic manipulation of the normally express enzyme in muscle using glucagon will provide and important degree of selectivity to the anti-tumor effect. This represents a novel use of selective promoter, taking advantage of its abnormal regulation in tumor cells.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

In vivo localization of gene distribution and expression

The pΔElspIBHex-LacZ may be used in tumor bearing rats for the *in vivo* localization of the suicide gene in pre-clinical testing of this novel targeting strategy. The gene construct is going to be administered in adenovirus type 5 recombinant vector or in lipid-based delivery system.

Materials and methods

Construction of recombinant viruses

Recombinant, replication deficient adenoviral vectors derived from type 5 adenovirus are constructed by the homologous recombination method in the human embryonic kidney cell line 293. The recombinant shuttle plasmids and pBHG11, containing the adenoviral genome, are co-transfected by calcium phosphate precipitation in 293 cells. The viral DNA is isolated from a single plaque and analyzed by restriction enzyme digestion. Recombinant adenovirus is expanded from a single plaque in 293 cells. Large scale production of the recombinant adenovirus is accomplished by growth in 293 spinner cells and purification by double cesium chloride gradient.

Results

These experiments are crucial to determine the best method of administration of the gene construct. It can either be done regionally to target specific organs such as the liver through portal vein injection or it can be administered intravenously. This method of looking at the distribution of the gene will allow us to determine the efficacy of uptake in the various organs and therefore establish a standard for use in humans.

EXAMPLE II

Targeted gene therapy for suicide destruction of tumors

The essential point is that the above-described HexII/VTK construct will be used in a vector/delivery system in clinical trials eventually.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any varia-

tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A tumor-specific promoter for use in gene targeted therapy that is differentially regulated in cancer cells, which comprises Hex II promoter.

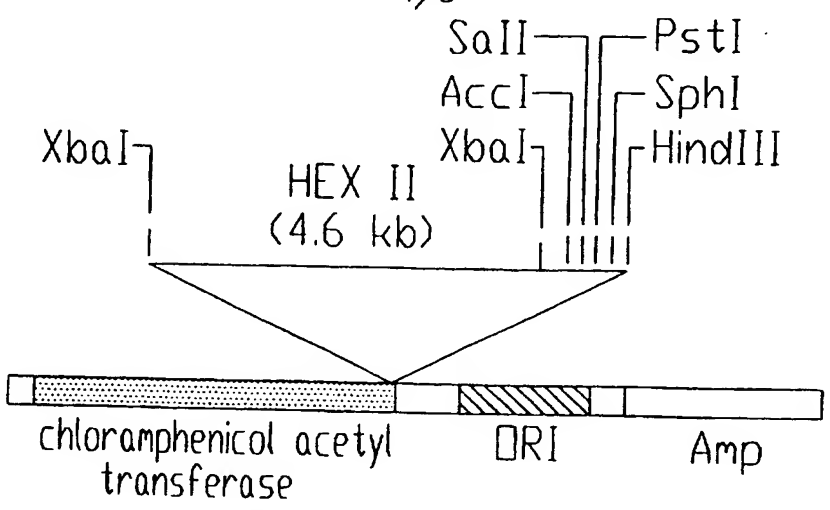
2. A Hex II gene construct, which comprises Hex II promoter in a vector selected from pCAT basic expression vector pΔElsplB and a shuttle plasmid.

3. The gene construct of claim 2, which further comprises β-gal or HSV Tk.

4. The gene construct of claim 2, wherein said vector is pCAT and said construct is pHexII4557-CAT.

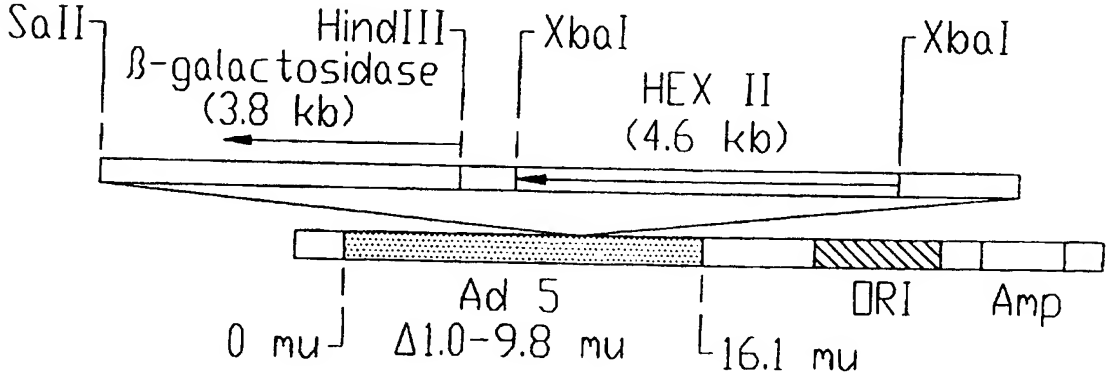
5. The gene construct of claim 3, wherein said vector is pΔElsplB and said construct is pΔElsplBHex-LacZ.

6. The gene construct of claim 3, wherein said vector is pΔElsplB and said construct is pΔElsplBHex-TK.



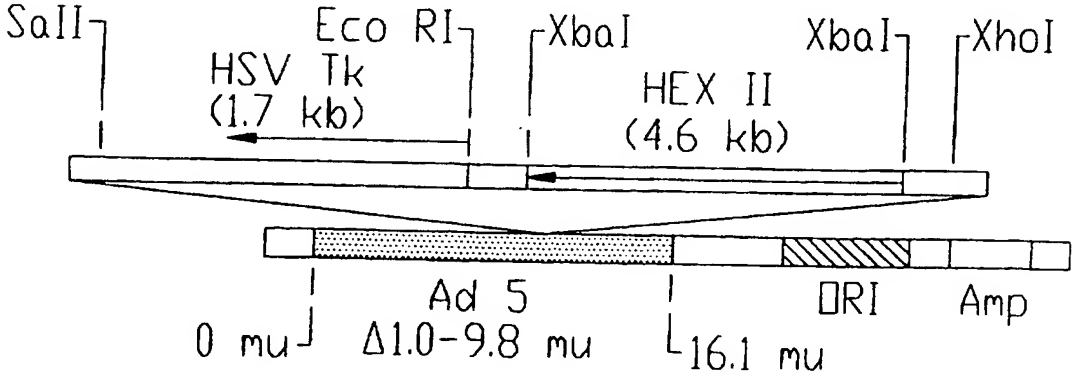
pHexII 4557/CAT

Fig. 1



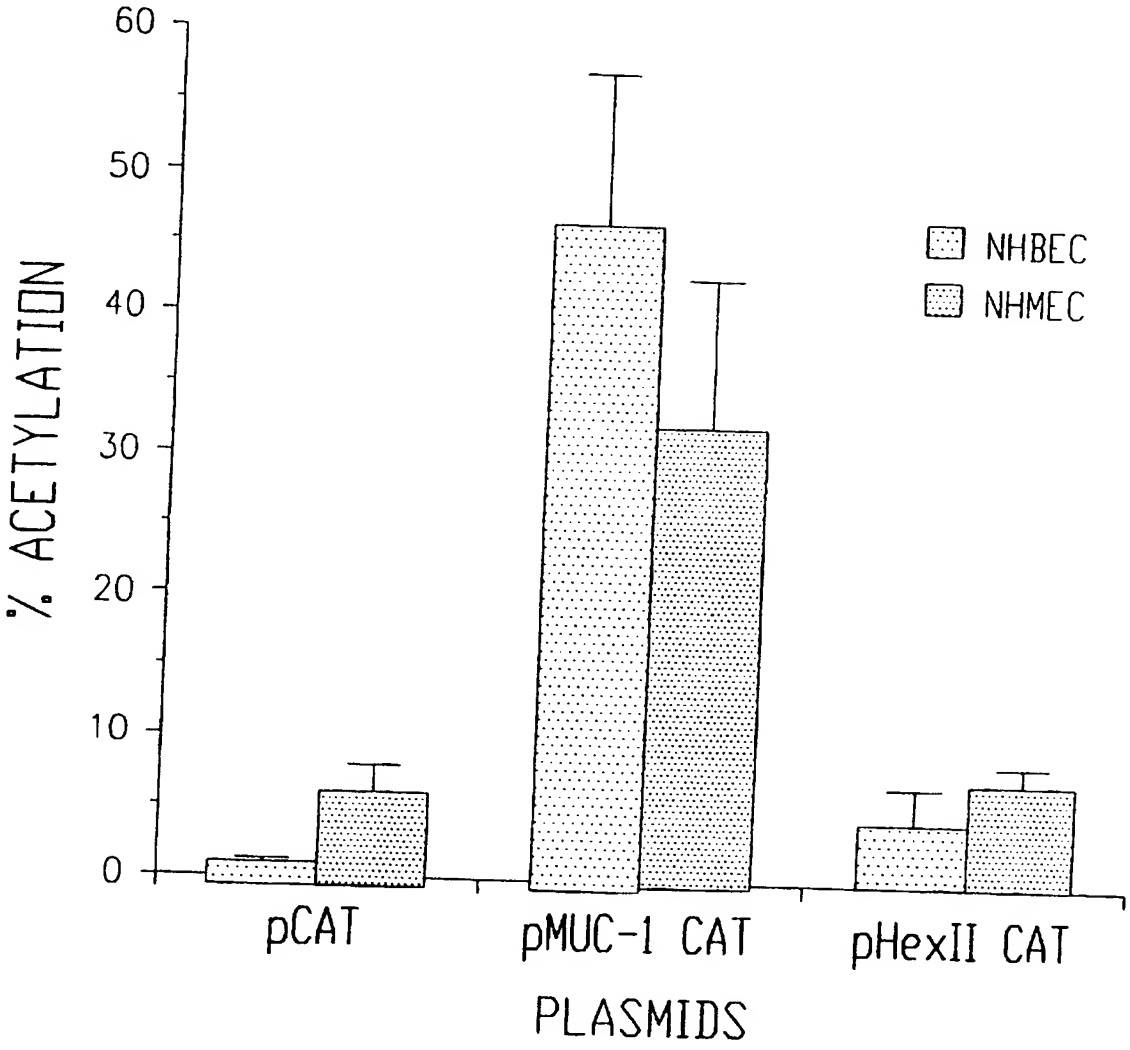
pΔE1sp1B HexII ß-galactosidase

Fig. 2



pΔE1sp1B HexII Tk

Fig. 3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40
30
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10
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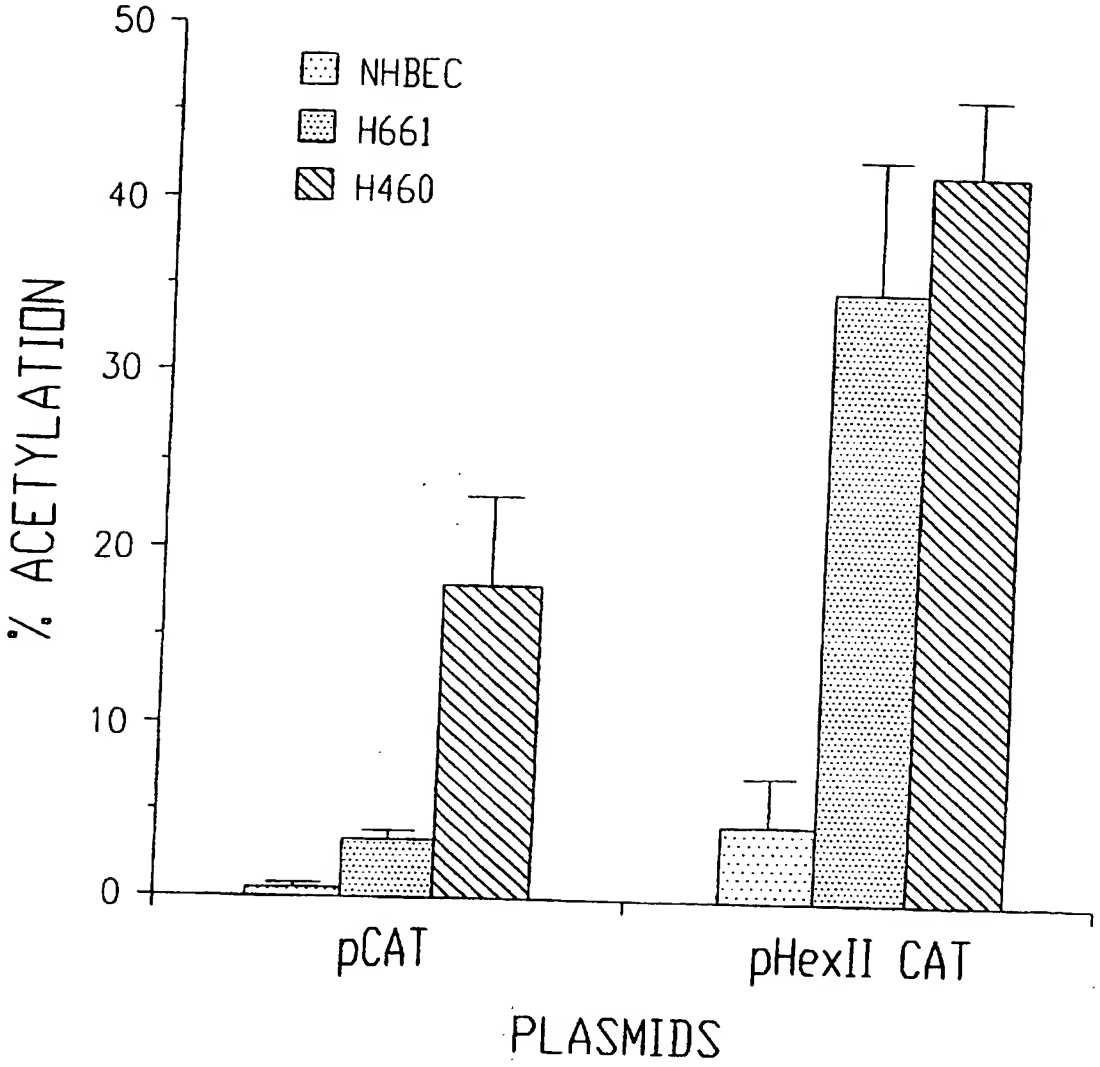
% ACETYLATION
NHBEC
NHMEC
pCAT
pMUC-1 CAT
pHexII CAT
PLASMIDS

Fig. 4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40
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% ACETYLATION
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H661
H460
pCAT
pHexII CAT
PLASMIDS

Fig. 5

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/CA 97/00691

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/54 C1201/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and where practical search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	MATHUPALA S. ET AL.: "Glucose catabolism in cancer cells" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 28, 14 July 1995, pages 16918-16925, XP002017888 cited in the application see the whole document ---	1
X	OSAWA H. ET AL.: "Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat hexokinase II gene" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 29, 19 July 1996, pages 17296-17303, XP002050907 see the whole document --- -/--	1-5

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

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Date of the actual completion of theinternational search	Date of mailing of the international search report
19 December 1997	15/01/1998

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSI[redacted]D TO BE RELEVANT

Category	Citation of document with indication where appropriate of the relevant passages	Relevant to claim No
A	REMPEL A. ET AL.: "Glucose metabolism in cancer cells: regulation of the Type II hexokinase promoter by glucose and cyclic AMP" FEBS LETTERS, vol. 385, no. 3, 6 May 1996, pages 233-237, XP002017889 see the whole document ---	1-6
A	HUBER B E ET AL: "VIRUS-DIRECTED ENZYME/PRODRUG THERAPY (VDEPT) SELECTIVELY ENGINEERING DRUG SENSITIVITY INTO TUMORS" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 716, 31 May 1994, pages 104-114, XP000654773 see the whole document ---	1-6
A	HARRIS J D ET AL: "GENE THERAPY FOR CANCER USING TUMOUR-SPECIFIC PRODRUG ACTIVATION" GENE THERAPY, vol. 1, no. 3, May 1994, pages 170-175, XP000654731 see the whole document ---	1-6
P,X	WO 97 04104 A (UNIV JOHNS HOPKINS ;PEDERSEN PETER LYNN (US); MATHUPALA SAROJ P (U) 6 February 1997 * see the whole document, esp. pp. 18-20, ex. 9 * -----	1-6

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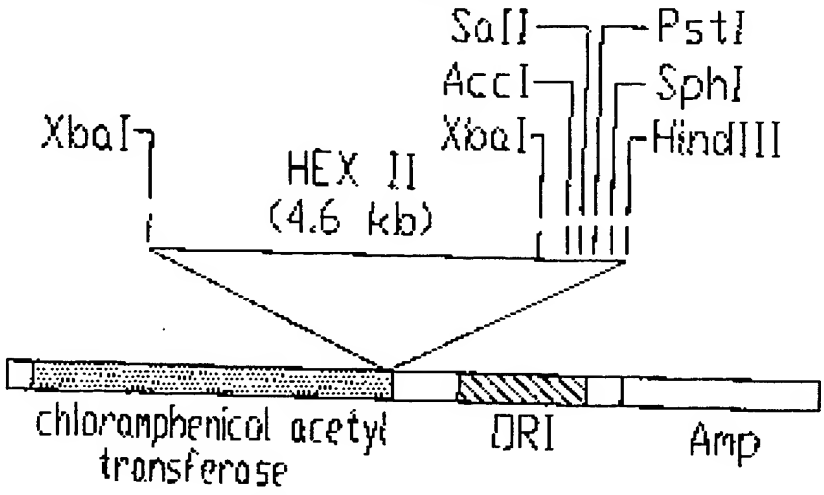
INTERNATIONAL SEARCH REPORT

nformation on patent family members

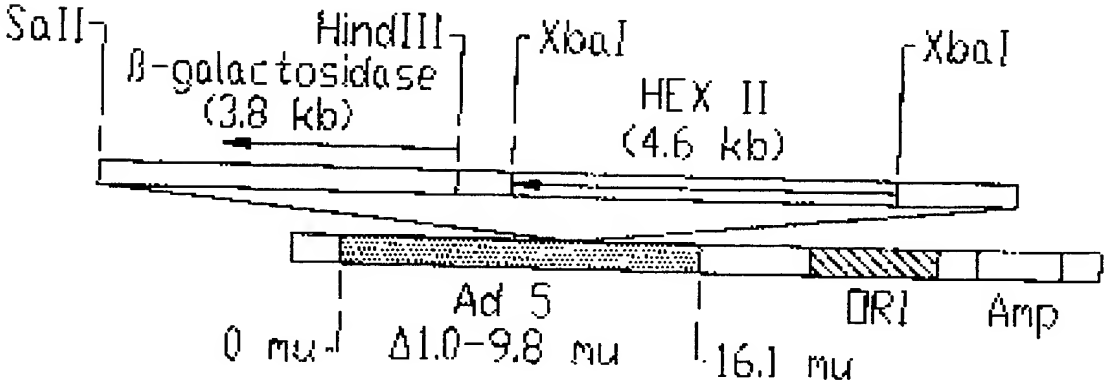
Int ional Application No
PCT/CA 97/00691

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9704104 A	06-02-97	AU 6676396 A	18-02-97



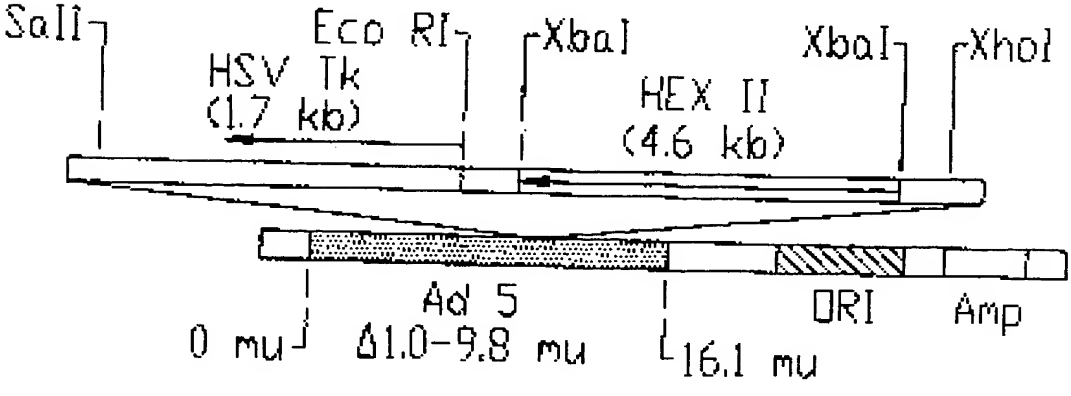
pHexII 4557/CAT

Fig - 1



pΔE1spIB HexII ß-galactosidase

Fig - 2



pΔE1spIB HexII Tk

Fig - 3

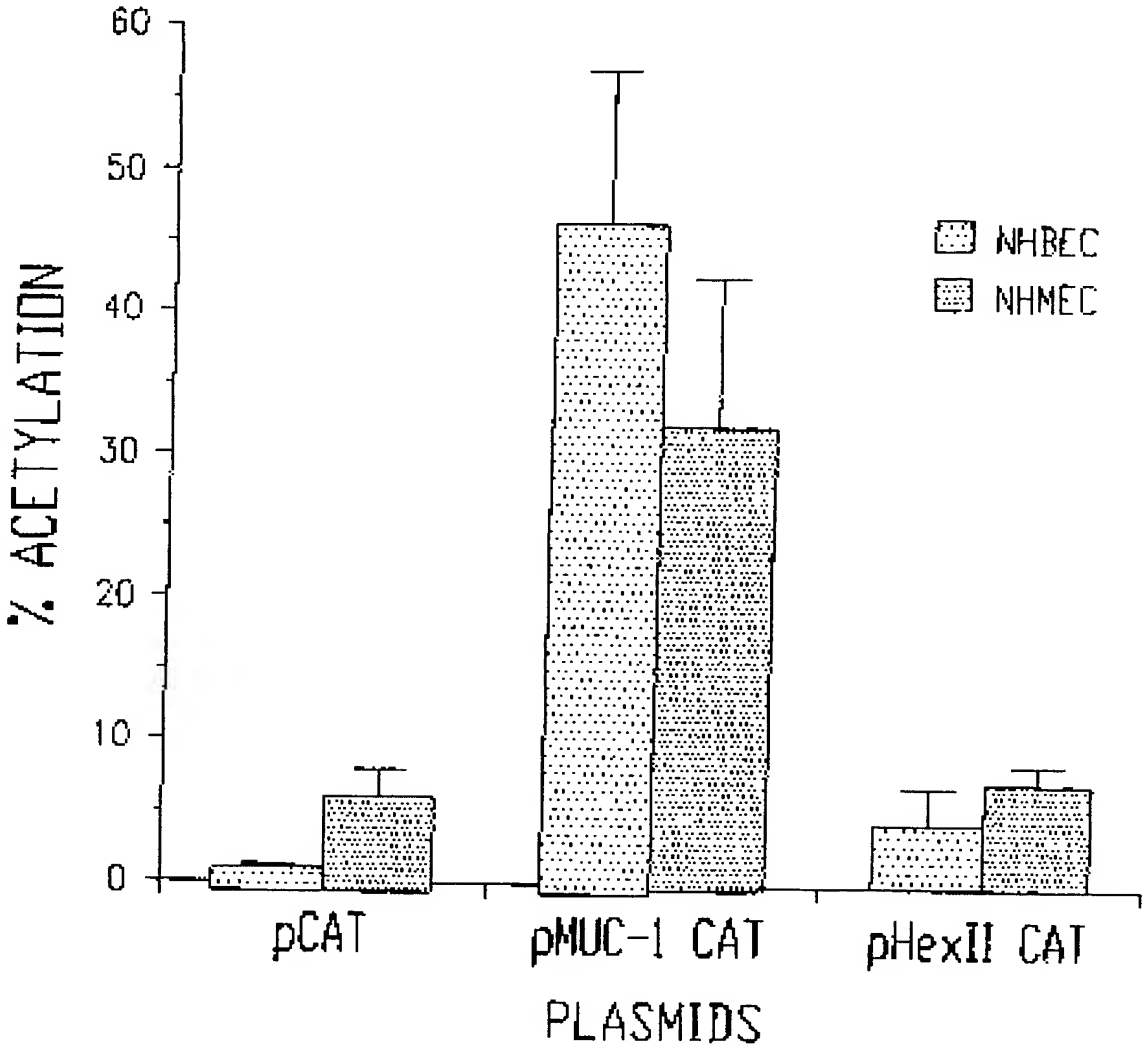


Fig. 4

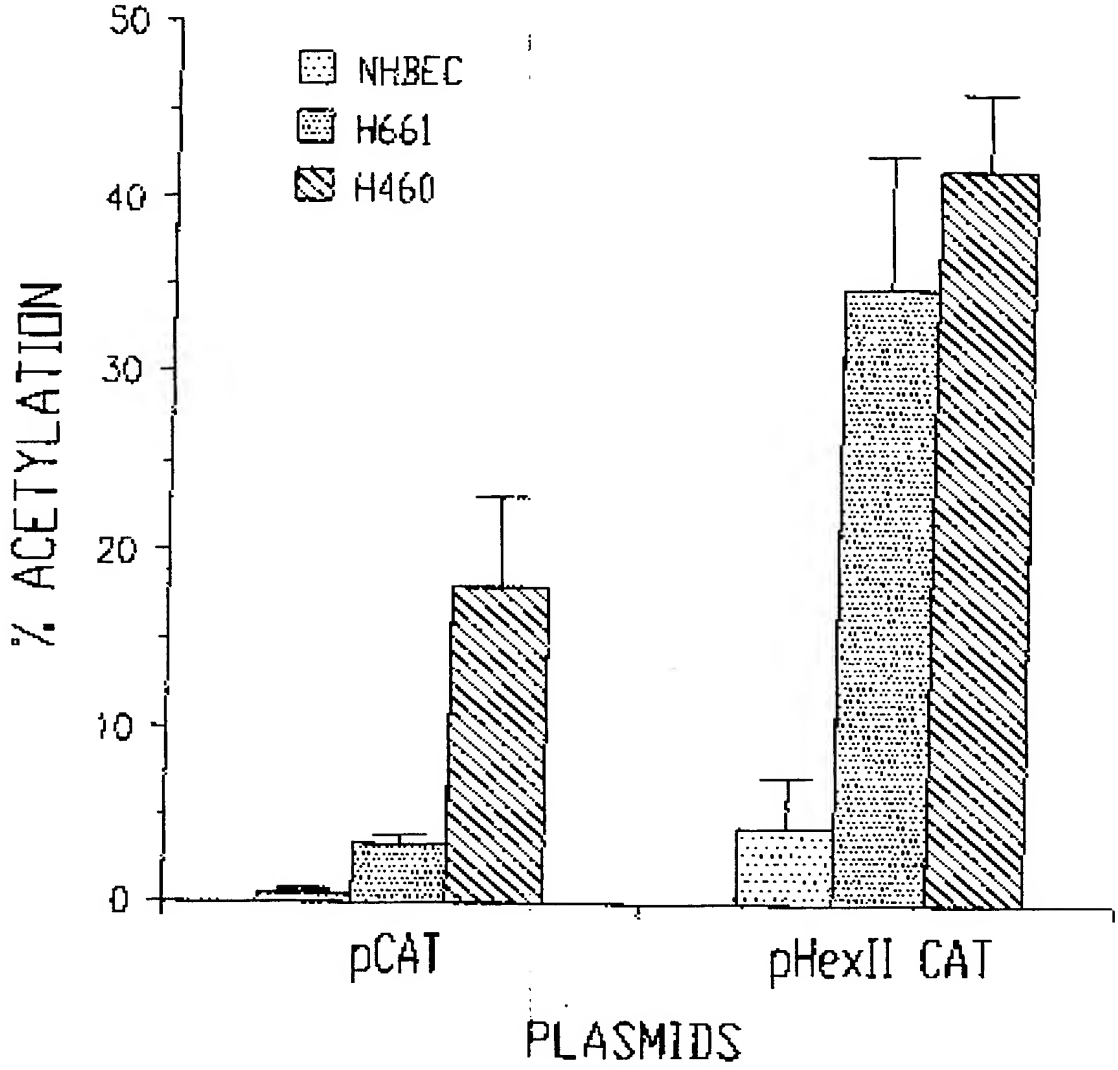

50
40
30
20
10
0
% ACETYLATION
NHBEC
H661
H460
pCAT
pHexII CAT
PLASMIDS

Fig. 5